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ABSTRACT

Prolonged exposure to estrogens is considered a major risk factor for development of breast cancer. When treated with estrogen for 28 weeks, ACI rats develop mammary cancers in over 90% of the population at risk. Genetic crosses between the susceptible ACI rat and resistant Copenhagen (COP) or Brown Norway (BN) rats identified a region on chromosome 5 (*Emca1*) that modified the development of estrogen-induced mammary cancer. To define the role of *Emca1* in the development of estrogen-induced mammary cancer, a congenic line has been developed (ACI.BN-*Emca1*) in which the resistant BN allele of *Emca1* has been introgressed onto an ACI background. Female ACI.BN-*Emca1* rats treated with estrogen for 28 weeks exhibit a significant decrease in the incidence of mammary cancer in the population at risk, a significant delay in the latency to the development of mammary cancer, and a significant decrease in the number of tumors per rat compared to ACI rats. These data suggest that *Emca1* is a strong modifier of estrogen-induced mammary cancer. Additional congenic sublines, in which the *Emca1* locus has been divided into smaller regions, have been generated and will be used to further define the region(s) on chromosome 5. In addition, microarray analysis of 12 week estrogen-treated mammary tissue from ACI and ACI.BN-*Emca1* rats was utilized to identify genes and ESTs that were differentially expressed as a result of estrogen treatment. Analysis of the ACI.BN-*Emca1* congenic sublines will be used to more clearly identify the region(s) on chromosome 5 that modify susceptibility to E2-induced mammary cancer, and microarray analysis will be used to identify genes that are differentially expressed as a result of E2 treatment. These data will provide important information on the mechanism(s) by which estrogen regulates the development of mammary cancer.

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INTRODUCTION: Prolonged exposure to estrogens is considered a major risk factor for development of breast cancer. When treated with the naturally occurring estrogen, 17β-estradiol (E2), ACI rats develop mammary cancers in over 90% of the population at risk. Genetic crosses between the susceptible ACI rat and resistant Copenhagen (COP) or Brown Norway (BN) rats identified a region on chromosome 5 (*Emca1*) that modified the development of estrogen-induced mammary cancer. The purpose of this research is to determine the role of *Emca1* in the development of mammary cancer. To define the role of *Emca1* in the development of mammary cancer, a congenic line was developed (ACI.BN-*Emca1*) in which the resistant BN allele of *Emca1* was introgressed onto an ACI background. During the first year of this grant, the response of ACI.BN-*Emca1* females to E2 was characterized. When treated with E2, the ACI.BN-*Emca1* congenic females develop mammary tumors with a significantly increased latency and a significantly decreased incidence in the population at risk. In addition, ACI.BN-*Emca1* females develop significantly decreased tumors per rat than the parental ACI females. Statistical and genotypic analysis was also employed in an attempt to localize the region of interest on chromosome 5. These data were utilized to develop four additional ACI.BN-*Emca1* congenic sublines.

This report summarizes progress during the second year of this grant.

BODY: The following accomplishments are documented according to the approved statement of work:

Task 1: Evaluate the impact of *Emca1* on the development of estrogen-induced mammary cancer.

During this reporting period, the response of mammary tissues from ACI, ACI.BN-*Emca1*, and BN rats following 12 weeks of E2 treatment was examined.

•**Experimental design**: Female ACI, BN and ACI.BN-*Emca1* rats were treated with E2 beginning at nine weeks of age. Following five weeks of treatment, animals were examined twice weekly for the presence of palpable mammary tumors. Mammary tissues were collected following 12 of E2 treatment.

• Female ACI (11), ACI.BN-*Emca1* (12), and BN (9) rats were treated with E2 for 12 weeks.

•**Results**: There was no difference in the mammary tissue from ACI, ACI.BN-*Emca1*, or BN rats sham-treated with empty implants for 12 weeks. No tumors were identified in any females following 12 weeks of E2 treatment. ACI and ACI.BN-*Emca1* females treated with E2 for 12 weeks exhibited no difference in the gross appearance of mammary glands at necropsy, and there was no discernible difference in whole mounts of mammary glands from the two rat strains. Importantly, there was also no difference histologically when comparing mammary tissue from 12 week E2-treated ACI and ACI.BN-*Emca1*. However, mammary tissue from 12 week E2-treated BN females was different from both ACI and ACI.BN-*Emca1* (Figure 1). These data suggest that *Emca1* does not affect the proliferative response of the mammary gland to estrogen. Instead, *Emca1* appears to delay the transformation of mammary hyperplasia to mammary cancer.

•**Status**: Because there was no discernable difference between the mammary glands from 12 week E2-treated ACI and ACI.BN-*Emca1* females, further analysis of differences in cell proliferation and focal regions of atypical hyperplasia will be completed during the final six months of the project, if applicable. Instead, 12 week E2-treated mammary tissue

is being utilized for analysis of changes in gene expression using the Affymetrix Rat 230 2.0 microarray chip (see Task 2 below).

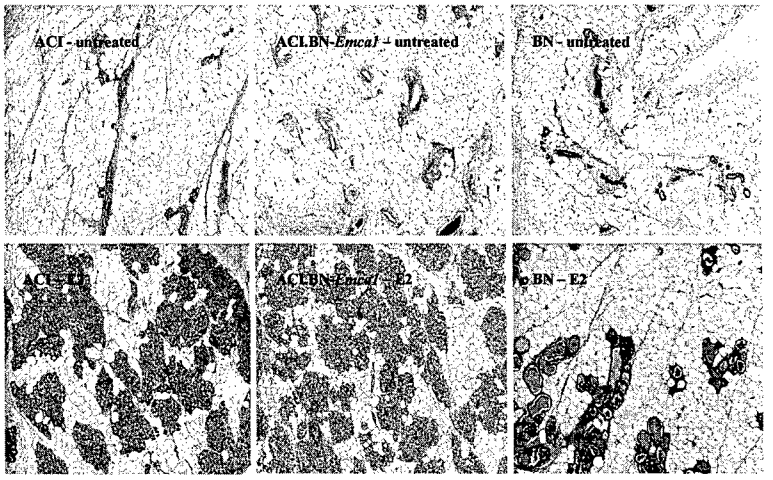


Figure 1. Rat strain-specific responsiveness of mammary tissue to 12 weeks of E2 treatment. The upper panel shows sham treated mammary tissue from ACI, ACI.BN-*Emca1*, and BN female rats. The bottom panel shows 12 week E2-treated mammary tissue from the corresponding strain of female rats.

Task 2: Establish more precisely the location of the genes that confer and/or modify susceptibility to estrogen-induced mammary cancer.

Additional genotyping and statistical analysis during the first year of this study did provide insight into regions on chromosome 5 that could modify E2-induced mammary cancer. These data were utilized to define regions of interest on chromosome 5 for the congenic sublines. However, because these analyses could not be utilized to more precisely define the genes within the *Emca1* locus that modify susceptibility to E2-induced mammary cancer, microarray analysis of 12 week E2-treated mammary tissue is being utilized to determine changes in gene expression and to identify genes within the *Emca1* locus that are differentially regulated by E2.

•**Experimental Design**: Total RNA was isolated from approximately 40 mg of 12 week E2-treated mammary tissue from ACI (5) and ACI.BN-*Emca1*(5) female rats using the Absolutely RNA miniprep kit from Stratagene. Two micrograms of RNA was labeled and hybridized to the Affymetrix Rat 230 2.0 microarray by personnel in the UNMC Microarray Core Facility.

•**Results**: Initial analysis of the microarray data eliminated those genes and expressed sequence tags (ESTs) that were not consistently present or absent in all five samples from a single rat strain. The remaining data were sorted and genes and ESTs were grouped according to the following categories: those present in the ACI, but absent in the ACI.BN-*Emca1*; those absent in the ACI, but present in the ACI.BN-*Emca1*; and those present in both strains and exhibiting at least a two-fold difference in expression. T-tests were performed on those genes and ESTs with at least a two-fold change to determine the significance of the change. These analyses identified a total of 40 genes or ESTs that were changed in the ACI compared to the ACI.BN-*Emca1* mammary tissue, of which 16 genes or ESTs reside on chromosome 5 within the *Emca1* locus (Table 1). A complete listing of the genes and ESTs summarized in Table 1 are included in Appendix I.

Table 1. Summary of changes in gene and EST expression in 12 week E2-treated mammary tissue.

Description	Total Number of Changes	Number of Changes on Chromosome 5
Absent ACI - Present ACI.BN-*Emca1*	3	0
Present ACI - Absent ACI.BN-*Emca1*	9	2
Present in both and:		
Increased ACI vs ACI.BN-*Emca1*	23	9
Decreased ACI vs ACI.BN-*Emca1*	5	5

These analyses indicate that microarray analysis can be utilized to identify genes and ESTs that are differentially expressed in the mammary tissue of different rat strains. These analyses provide important tools to identify genes and ESTs, both globally and within *Emca1*, that are differentially expressed as a result of E2 treatment. In addition, other methods, such as quantitative real time PCR, will be utilized to verify the expression of selected genes.

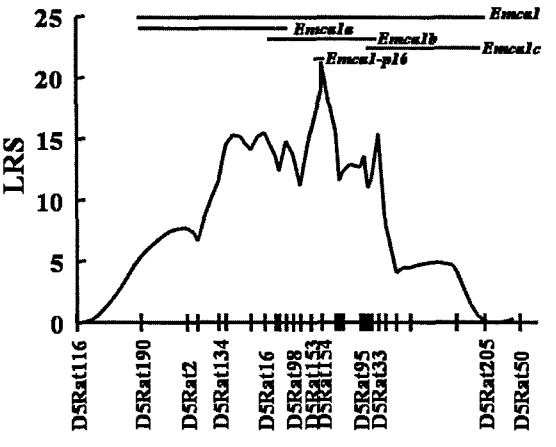
• **Status**: Microarray analysis of 12 week E2-treated mammary tissue from BN rats and matched untreated mammary tissue for ACI, ACI.BN-*Emca1*, and BN strains will be completed during the final year. Comparison of gene expression profiles of mammary tissue from treated and untreated rats will provide a comprehensive list of genes and ESTs that are differentially expressed as a result of E2 treatment in the susceptible ACI and resistant BN, including those genes and ESTs within *Emca1*. Identifying the estrogen responsive genes will provide insights into the mechanism of estrogen action in the development of mammary cancer.

Task 3: Characterize additional congenic lines carrying specific intervals of *Emca1* to determine the effect of genotype on susceptibility to estrogen-induced mammary cancer.

During this reporting period, a fourth *Emca1* congenic subline was completed and female rats from all sublines are currently being treated with E2.

• **Experimental Design**: Treat females from the four *Emca1* congenic sublines (ACI.BN-*Emca1a*, ACI.BN-*Emca1b*, ACI.BN-*Emca1c*, and ACI.BN-*Emca1-p16*; Figure 2) with E2 for 28 weeks beginning at nine weeks of age.

Figure 2. Boundaries of the four *Emca1* congenic sublines. The *Emca1* locus has been divided and multiple congenic lines developed to better define the region of interest on chromosome 5. Lines represent the region on chromosome 5 encompassed by the congenic sublines.



• **Results**: Because only a limited number of rats from each *Emca1* subline have been treated for the full 28 weeks, incidence in the population at risk was analyzed following 20 weeks of E2 treatment, which represents the mean and median latency to the development of mammary tumors for the susceptible ACI rat. Following 20 weeks of E2 treatment, all *Emca1* congenic sublines exhibited reduced incidence of mammary tumors in the population at risk (Table 2), suggesting that multiple modifiers of E2-induced mammary cancer resides within the *Emca1* locus. However, a larger population of rats from each congenic subline, treated for the full 28 weeks, is required to determine whether or not a modifier of E2-induced mammary cancer resides within the region of *Emca1* found in these congenic sublines.

Table 2. Incidence of mammary tumors in the population at risk following 20 weeks of E2 treatment.

Strain	Number of Rats Treated for 20 Weeks	Percent Incidence Following 20 Weeks E2
ACI	80	50.4
ACI.BN-*Emca1*	41	4.9
ACI.BN-*Emca1a*	16	0
ACI.BN-*Emca1b*	25	13.6
ACI.BN-*Emca1c*	32	3.2
ACI.BN-*Emca1-p16*	9	11.1

• **Status**: Additional females from each of the congenic lines will be treated with E2, with a goal of treating 40 rats from each of the congenic sublines.

KEY RESEARCH ACCOMPLISHMENTS:

• Analysis of the proliferative response of the ACI.BN-*Emca1* congenic line
• Initial analysis of changes in gene expression in the ACI.BN-*Emca1* congenic line compared to the parental ACI (APPENDIX I)
• Initiation of E2 treatment for the ACI.BN-*Emca1* (26 rats), ACI.BN-*Emca1b* (35 rats), ACI.BN-*Emca1c* (32 rats), and ACI.BN-*Emca1-p16* (10 rats)

REPORTABLE OUTCOMES:

Gould, K.A., Murrin, C.R., Flood, L.A., Pennington, K.L., Schaffer, B.S., Tochacek, M., McComb, R., Meza, J.L., Wendell, D., and Shull, J.D. Genetic mapping of *Eutr1*, a locus controlling E2-induced pyometritis in the Brown Norway rat, to RNO5. *Mammalian Genome*. 2005 (accepted)

Schaffer, B.S., Tochacek, M., Pennington, K.L., Meza, J.L., and Shull, J.D. Evidence that *Emca1* is a genetic determinant of E2-induced mammary tumor incidence and tumor multiplicity in the

ACI rat. Era of Hope – Department of Defense Breast Cancer Research Program Meeting, 2005 (Oral and Poster)

Gould, K.A., Tochacek, M., Schaffer, B.S., Reindl, T.M., Murrin, C.R., Lachel, C.M., VanderWoude, E.A., Pennington, K.L., Flood, L.A., Bynote, K.K., Meza, J.L., Newton, M.A. and Shull, J.D. Genetic Determination of Susceptibility to Estrogen-Induced Mammary Cancer in the ACI Rat: Mapping of *Emca1* and *Emca2* to Chromosomes 5 and 18. *Genetics.* 2004 Dec; 168(4):2113-25.

Strecker, T.E., Spady, T.J., Kaufman, A.E., Shen, F., McLaughlin, M.T., Pennington, K.L., Meza, J.L., Schaffer, B.S., Gould, K.A., and Shull, J.D. Genetic Bases of Estrogen-Induced Pituitary Tumorigenesis: Identification of Genetic Loci Determining Estrogen-Induced Pituitary Growth in Reciprocal Crosses between the ACI and Copenhagen Rat Strains. *Genetics.* 2005 Apr; 169(4):2189-97. .

Schaffer, B.S., McLaughlin, M.T., Tochacek, M., Pennington, K.L., Meza, J.L. and Shull, J.D. 2004 Confirmation of *Emca1*, a locus that modifies development of estrogen-induced mammary tumors, in the ACI.BN-*Emca1* congenic rat strain. XVth International Workshop on Genetic Systems in the Rat (Oral).

Schaffer, B.S., McLaughlin, M.T., Tochacek, M., Pennington, K.L., Meza, J.L., McComb, R.D. and Shull, J.D. 2004 Characterization of estrogen-induced mammary cancer in the ACI.BN-*Emca1* congenic rat: evidence that *Emca1* inhibits mammary carcinoma. San Antonio Breast Cancer Symposium (Poster).

CONCLUSTIONS:

The ACI.BN-*Emca1* congenic line exhibited delayed latency to the development of E2-induced mammary cancer, decreased incidence within the population at risk, and decreased tumors per rat when compared with the susceptible ACI parental rats. Characterizing the susceptibility of the additional congenic lines developed in Task 3 will further refine the *Emca1*interval and aid in identifying the genes within *Emca1* that modify susceptibility to E2-induced mammary cancer. Analysis of gene expression profiles, utilizing Microarray technology, will provide a global list of those genes differentially modified as a result of estrogen treatment. Together, these analyses will provide unique insight into the mechanisms of estrogen action in the development of mammary cancer. Understanding how estrogen promotes the development of mammary cancer in the ACI rat could lead to the development of novel treatments, to prevent the development of breast cancer and to treat existing breast cancer, in humans.

Absent ACI - Present *Emca1*

Affy ID	Location	Descriptions
1392230_at	chr1:190388300-190388723 (+) // 97.64 //	TITLE=ESTs
1394806_at	chr1:83732368-83732850 (-) // 98.96 //	TITLE=ESTs
1387718_at	chr12:34937411-34980540 (-) // 98.9 //	Rattus norvegicus purinergic receptor P2X, ligand-gated ion channel, 7 (P2rx7), mRNA.

Present ACI - Absent Emca1

Affy ID	Location	Descriptions
1384380_at	**chr5:61462857-61463366 (+) // 96.39 //**	**TITLE=ESTs**
1374527_at	chr12:19761418-19762265 (-) // 32.8 // /// ch	TITLE=ESTs, Weakly similar to ENOYL-COA HYDRATASE, MITOCHONDRIAL PRECURSOR (R.norvegicus)
1383575_at	chr2:83033361-83033862 (+) // 95.38 //	TITLE=ESTs
1384049_at	chr9:18069442-18075545 (+) // 96.84 //	TITLE=ESTs, Highly similar to AP2B_MOUSE Transcription factor AP-2 beta (AP2-beta) (Activating enhancer-binding protein 2 beta) (M.musculus)
1368677_at	chr3_random:930775-979584 (+) // 87.68 //	Rattus norvegicus Brain derived neurothrophic factor (Bdnf), mRNA.
1376692_at	chr4:66550710-66551313 (-) // 89.22 //	TITLE=ESTs, Weakly similar to homeodomain-interacting protein kinase 3 (Rattus norvegicus) (R.norvegicus)
1376789_at	---	TITLE=ESTs
1381120_at	**chr5:65123670-65124140 (+) // 99.36 //**	**TITLE=ESTs**
1370086_at	chr2:174677399-174684707 (+) // 94.08 //	Rattus norvegicus Fibrinogen, gamma polypeptide (Fgg), mRNA

Increased in ACI compared to *Emca1*

Affy ID	Location	ACI Mean	*Emca1* Mean	Fold Change	t-test	Descriptions
1397168_at	**chr5:145517809-145518662 (+) // 94.43 //**	**1628.5**	**313.4**	**5.1962348**	**0.001016**	**TITLE=ESTs**
1398241_a_at	chr7:142825519-142837126 (-) // 95.92 // ///	3402.28	795.56	4.276585	0.002418	Rattus norvegicus salivary protein 1 (Spt1), mRNA.
1393494_at	chr12:16202790-16203282 (-) // 93.14 //	895.1	235.5	3.8008493	0.129017	TITLE=ESTs
1372621_at	**chr5:145514197-145514715 (+) // 96.62 //**	**7746.42**	**2549.48**	**3.0384314**	**0.001119**	**TITLE=ESTs**
1392736_at	---	8622.24	2893.3	2.9800712	0.001968	TITLE=ESTs
1384799_at	chrX:92152645-92152990 (-) // 95.04 //	421.38	147.2	2.8626359	0.03825	TITLE=ESTs, Weakly similar to T17202 DNA-directed DNA polymerase (M.musculus)
1381353_at	chrX:150038616-150039019 (+) // 95.72 //	728.34	255.08	2.8553395	0.008088	TITLE=ESTs
1377939_at	**chr5:119898113-119898626 (+) // 99.03 //**	**660.46**	**258.74**	**2.5526011**	**0.002898**	**TITLE=ESTs**
1371993_at	**chr5:34154848-34156468 (-) // 83.86 //**	**1245.92**	**510.9**	**2.4386768**	**0.000299**	**TITLE=ESTs, Weakly similar to CNE6_MOUSE COPINE VI (NEURONAL-COPINE) (N-COPINE) (M.musculus)**
1398431_at	**chr5:21951217-21951671 (-) // 98.91 //**	**2707.08**	**1147.96**	**2.3581658**	**0.000793**	**TITLE=ESTs, Weakly similar to CAH2 RAT CARBONIC ANHYDRASE II (R.norvegicus)**
1368048_at	chr6:128383137-128390546 (-) // 97.81 // ///	1007.36	432.48	2.3292638	0.015762	Rattus norvegicus Serine protease inhibitor (Spin2b), mRNA. /PROD=serine protease inhibitor 2b /FL=gb:NM_012657.1
1378315_at	**chr5:21948815-21949253 (-) // 100.0 //**	**1270.68**	**556.52**	**2.2832603**	**0.000346**	**TITLE=ESTs**
1371677_at	chr8:114210076-114221630 (+) // 91.68 //	2904.52	1291.02	2.249787	1.19E-05	TITLE=ESTs, Weakly similar to I52196 homeobox transcription factor Hox 1.11 - rat (R.norvegicus)

1379497_at	---	24434.96	10931.92	2.2351938	0.007693	TITLE=ESTs
1395142_at	chrX:92143480-92143959 (-) // 98.54 //	736.46	331.54	2.2213308	0.017087	TITLE=ESTs
1389118_s_at	chr2:182751292-182751684 (+) // 95.61 //	1908.64	875.98	2.1788625	0.000492	TITLE=ESTs, Weakly similar to S113 MOUSE S100 CALCIUM-BINDING PROTEIN A13 (M.musculus)
1379425_at	**chr5:141512914-141513361 (-) // 97.11 //**	**420.7**	**193.44**	**2.1748346**	**0.002661**	**TITLE=ESTs**
1373352_at	chr2:182751292-182751684 (+) // 95.61 //	1022.12	497.92	2.0527796	0.000663	TITLE=ESTs, Weakly similar to S113 MOUSE S100 CALCIUM-BINDING PROTEIN A13 (M.musculus)
1368731_at	**chr5:80330154-80333307 (+) // 98.46 //**	**27698.3**	**13512.52**	**2.0498249**	**0.019887**	**Rattus norvegicus Orosomucoid 1 (Orm1), mRNA.**
1387631_at	chr16:37420793-37457987 (-) // 99.54 //	6591.34	3244.1	2.0317931	0.005787	Rattus norvegicus NAD-dependent 15-hydroxyprostaglandin dehydrogenase (Hpgd), mRNA.
1385014_at	---	327.08	161.58	2.0242604	0.029225	TITLE=EST
1379582_a_at	chr2:123007825-123008297 (-) // 99.36 // ///	909.62	450.12	2.0208389	0.000957	TITLE=ESTs, Weakly similar to CGB1 RAT G2MITOTIC-SPECIFIC CYCLIN B1 (R.norvegicus)
1380144_at	chr14:20910867-20911417 (-) // 97.5 //	442.36	219.22	2.0178816	0.024771	TITLE=ESTs
1369044_a_at	**chr5:123356922-123538838 (+) // 99.05 //**	**1756**	**874.72**	**2.0074995**	**0.000452**	**Rattus norvegicus cAMP-specific phosphodiesterase isoform PDE4B4 (Pde4b) mRNA, complete cds**

Decreased in ACI compared to *Emca1*

Affy ID	Location	ACI Mean	*Emca1* Mean	Fold Change	t-test	Descriptions
1391791_at	**chr5:106238412-106239036 (+) // 88.76 //**	**180.7**	**378.92**	**0.467275**	**0.0504**	**TITLE=ESTs, Weakly similar to T32993 hypothetical protein W02F12.2 - Caenorhabditis elegans (C.elegans)**
1393613_at	**chr5:72967074-72967602 (+) // 99.05 //**	**271.9**	**767.48**	**0.418486**	**0.015179**	**TITLE=ESTs**
1373302_at	**chr5:106242158-106242826 (+) // 98.21 //**	**388.7**	**1031.34**	**0.399694**	**0.003087**	**TITLE=ESTs**
1371960_at	**chr5:151073996-151074527 (+) // 94.14 //**	**1098.8**	**3731.9**	**0.365610**	**0.049884**	**TITLE=ESTs**
1382431_at	**chr5:70495509-70496084 (-) // 98.96 //**	**1540.2**	**3602.2**	**0.295036**	**0.004831**	**TITLE=ESTs**

List of genes and ESTs modified in mammary tissue following 12 weeks of E2 treatment. Those genes and ESTs in bold are within the *Emca1* region on chromosome 5.